

Isolation of Host Proteins Involved in Herpes Simplex Virus Type 1 DNA Synthesis

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By
Whitney Cogswell

The Ohio State University
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Advisor: Professor Deborah Parris, Department of Molecular Genetics

Abstract

Herpes simplex virus type 1 (HSV-1) is the prototypic member of a large family of viruses known as Herpesviridae. Herpesviruses possess a double-stranded DNA genome and infect a broad range of species. In addition to HSV-1, there are seven other herpesviruses that are known to infect humans. Although antiviral treatment is available for HSV infections, there are few or no options for treatment of infections by other herpesviruses. One of the best targets for development of new antivirals is the viral DNA replication apparatus. HSV-1 encodes for seven proteins directly involved in viral DNA replication. However, no HSV-1 origin-dependent DNA replication has been reconstituted in vitro using purified viral proteins. It is hypothesized that proteins (host and/or viral) in addition to the seven known viral DNA replication proteins are involved in viral DNA replication in vivo. Moreover, it is predicted that such proteins would interact with one or more of the viral DNA replication proteins. In an effort to identify interacting host proteins, co-immunoprecipitation analysis was performed using antibodies specific for an essential HSV DNA replication protein, UL42, the polymerase processivity factor. Extracts from infected and mock-infected cells were immunoprecipitated with the UL42 antibody. UL42, and all that is bound to it, bind to the antibody which binds to the Protein G agarose matrix. Those proteins that are specifically pulled down from infected cell extracts, but not from mock-infected extracts, were to be identified by mass-spectrometry. The immunoprecipitates were analyzed following separation on denaturing polyacrylamide gels. Gels were silver-stained or analyzed for the presence of UL42 using a Western blot. Conditions were adjusted to optimize the amount of UL42 immunoprecipitated, but UL42 could not be immunoprecipitated quantitatively.

Following immunoprecipitation under optimum conditions, no differences were observed between the proteins pulled-down from mock- or infected-cell extracts. Due to the poor ability of the antibody to pull-down UL42, a second approach was employed. A histidine-tagged version of UL42 was purified using a nickel chelating column and then mixed with infected or mock-infected cell extracts. Those proteins that specifically interact with his-UL42 were expected to bind to a nickel column. However, nonspecific interactions with the matrix were observed and prevented identification of proteins by mass-spectrometry.

The Family Herpesviridae

The family of viruses known as Herpesviridae is a large one that includes pathogens of species ranging from mollusks to humans (21). All herpesviruses can cause asymptomatic infections and remain latent within their respective hosts (21). Even years after an initial infection, the host can have reoccurring episodes of disease. For many of the viruses, the tendency is to infect one type of epithelial cell and to establish latency in a different nonepithelial cell type. There are hundreds of herpesviruses, but only eight are endogenous to humans (14). In order to better characterize such a diverse family, Herpesviridae has been divided into three subfamilies: alpha, beta, and gamma herpesviruses (15). These subfamilies were initially separated based on patterns in biological properties, such as tissue and host specificity during latent and active stages of infection, range of host cell infection in tissue cultures, and duration of infection. Now it is known that the group assignments correlate with similar genetic content and organization. Much is conserved genetically within each subfamily. For example, genes that are similarly regulated or expressed at similar stages are also clustered together within the genome at similar locations and with similar orientations. In addition to comparable genetic organization, each group also shows significant amino acid homology throughout their corresponding protein structures (18).

The alphaherpesviruses infect the broadest range of hosts in the Herpesviridae family (14, 15). They establish latency in neuronal and glial cells. Also, these viruses have a short replication cycle, so they are able to spread rapidly (18). Examples of

viruses in this subfamily include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) as well as the varicella zoster virus (VZV) (14).

HSV-1 is ubiquitous in the human population (19). Approximately 80 to 90 percent of adults will be infected by HSV-1 within their lifetimes. HSV-1 causes cold sores, fever blisters, and, less commonly but more critically, encephalitis. In the United States, HSV-2 is responsible predominantly for genital herpes, though it can also be associated with oral lesions. It is less widespread as it is found in only 20 to 50 percent of adults. Both types of HSV are primarily transmitted by direct contact with infected material (usually in body fluids) through mucosal membrane (4). Herpes labialis, the most prevalent symptom of HSV-1 infection, occurs on the cheek and gum areas with ulcerations or lesions often present on the lip (1). Herpes genitalis is usually the result of an HSV-2 infection; however, HSV-1 can also lead to mild symptoms in this anatomical area (1). Herpesvirus infections of neonates can occur when a woman with an active genital herpes infection delivers child (1). Severe consequences, ranging from symptomatic lesions to encephalitis, can occur if the neonate is not treated. Rarer symptoms of adult HSV infections may occur in the eyes, fingers, or toes.

Upon initial (primary) infection, the virus begins its replication cycle within epidermal cells, leading to cell death (1). Some of the infected cells lyse, and fluid accumulates between the cell layers. This produces the visible lesions and ulcerations. The virus can also get into neuronal cells that innervate the epithelial layer. However, HSV-1 does not replicate in these cells. The genome is maintained leading to a state of latency.

VZV, also known as human herpesvirus 3, is another common alphaherpesvirus that is usually contracted in childhood (19). As many as 50 to 75 percent of children have been infected with the virus, and up to 85 to 95 percent of adults harbor latent VZV. VZV is responsible for the familiar childhood illness, chickenpox, which is the outcome of a primary infection (19). Because VZV, like all herpesviruses, can remain latent in their hosts, VZV can go unnoticed for years and then become active again. This reoccurring episode of illness (reactivation) is known as shingles. Shingles presents itself as a painful, burning rash on the host's skin. As with many herpesviruses, there is some trigger that causes the virus to return from latency. It is suspected that one trigger is stress. It is known that the virus often returns in elderly patients or patients who have become immunocompromised. This means that the immune system, for one reason or another, helps to control the virus during the latent period.

VZV is unique in that it is the only herpesvirus for which a vaccine is readily available (19). There have been many attempts to create a vaccine for HSV (2). Though several of the vaccinations appeared to be safe, the benefits seemed to be minimal. Most active HSV and VZV infections are treated with antiviral medications (4). The goal of such medication is to stop the replication cycle as soon as possible. The best way to achieve that is to target viral replication in a way that will not harm the host cells. There are a few types of agents currently available for treatment of HSV infections. Acyclovir, valacyclovir, penciclovir, and famciclovir produce similar active compounds once they are phosphorylated by the viral thymidine kinase. Acyclovir is a nucleoside analogue drug that competes with dGTP for its binding spot on the viral

polymerase and terminates chain elongation if it is added to DNA (1). The problem with acyclovir is its short half life. Valacyclovir has a longer half life in the blood stream, so it can be taken less often. It works by the same mechanism because it is simply metabolized into acyclovir. In their active forms, famciclovir and penciclovir also work to inhibit the viral DNA polymerase by similar mechanisms. Foscarnet, a phosphonoformate, is a different inhibitor of the viral DNA polymerase that binds to the pyrophosphate binding site (1, 4). Because of its high toxicity, it is usually administered in a hospital setting. Foscarnet is used primarily to treat acyclovir-resistant infections.

Betaherpesviruses are not as well studied as the alphaherpesviruses. They infect a narrower range of hosts, establish latency in lymphoreticular or hematopoietic cells, and cause mostly asymptomatic infections (14, 18, 21). These viruses can be distinguished by their tendency to cause infected cells to enlarge. Examples include human cytomegalovirus (HCMV) and human herpes viruses 6 and 7 (HHV-6 and HHV-7) (14).

CMV is also called human herpesvirus 5 (18). Most people have contracted CMV at one time or another, but many will never show symptoms (19). In fact, about 80 to 100 percent of adults will test positive for CMV by the time they reach age 70. However, if infection is symptomatic, it can be very serious (19). Symptoms include fever, swollen glands, and fatigue. CMV is unique as it can be passed from mother to child in utero (transplacentally) while the other herpes viruses can only be contracted during birth (18). If spread this way, the child is at risk for some congenital birth defects, including mental retardation or deafness.

HHV-6 has ties with many symptoms and diseases (16). The illness exanthema subitum, also known as roseola infantum (18), is caused by HHV-6 and occurs mostly in children. A high fever and rash characterize the disease. In more severe cases, children may also experience seizures. Children are usually infected with HHV-6 by age two or three (16). Infection is almost universal in the adult population. Studies have also linked HHV-6 to certain types of lymphoma and multiple sclerosis (16). Even associations with hepatitis and chronic fatigue syndrome have been noted (17).

HHV-7 is typically contracted later in childhood than HHV-6, but it is still found in close to 95 percent of adults (16). It is closely related to HHV-6, and they were discovered around the same time (19). Like HHV-6, HHV-7 is connected with exanthema subitum which causes fever, rash, and possible seizures (16). HHV-7 has some correlation with mononucleosis as well.

The gammaherpesviruses are separated into two groups: lymphocytoviruses and rhadinoviruses (14, 15). They replicate in epithelial cells and establish latency in lymphocytes. Examples in this subfamily include the Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV-8). As a group, these viruses cause the most severe diseases of all the herpesviruses.

EBV is acquired at a later age than most of the other human herpesviruses, but 80 to 95 percent of adults carry it (19). It is the causative agent for most forms of infectious mononucleosis (18, 19). EBV has also been shown to be associated with Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease. There is a high incidence of disease caused by EBV in equatorial Africa (26). Many people in this

region have risk factors such as AIDS and malaria, so their immunosuppressed state makes them susceptible to EBV.

HHV-8 is also known as Kaposi sarcoma associated herpesvirus because of its connection to Kaposi sarcoma (16, 18). Though Kaposi sarcoma is the usual outcome of infection, HHV-8 has also produced proliferative skin lesions and led to Castleman disease (17). HHV-8 was the most recent human herpesvirus to be discovered (17). Tissue samples were taken from Kaposi sarcoma lesions of patients as well as normal tissue in order to analyze the cause of the disease. Comparison between the mRNA within these samples demonstrated sequences homologous to known herpesviruses. Since that time, the virus has been isolated and is the eighth human herpesvirus. The distribution of this virus is limited and uneven especially when compared to the almost universal infections with the other herpesviruses (25). HHV-8 is found in only 5 to 10 percent of adults (19). However, it is found in 70 to 100 percent of those with Kaposi sarcoma which is consistent with the relationship of virus to the disease. Pockets of viral infection and Kaposi sarcoma can be seen in sub-Saharan Africa and in the Mediterranean areas (25). The disease is unusual in that it is only found in men. HHV-8 is also more prevalent among those with HIV especially throughout the United States.

HSV-1 is considered the prototype for human herpesviruses and is used as a model to understand the basics of the replication cycle of the other viruses (14). There are several benefits to using HSV-1 as a model. First, HSV-1 infects a broad range of hosts rather than just infecting a single species (21). Second, HSV-1 also has a short replication cycle in tissue culture and can produce large quantities of virus (18).

Conveniently, the proteins necessary and sufficient for DNA replication in HSV-1 are encoded by its own genome (11).

Basis Steps of HSV-1 Replication Cycle

It is the ultimate goal of any virus to enter a host system, replicate, and spread (20). The virus wants to deliver its contents to the cell in replication competent form with little damage to the host so that there is no detection from the immune system. Herpesviruses are composed of a capsid that encloses a DNA genome (21, 22). The tegument is a layer of proteins that coats the capsid. The tegument is surrounded by an envelope composed of viral proteins and glycoproteins in a lipid bilayer. Upon infection, the virus must attach to and enter the host cell (20). The genome then needs to be uncoated from its protective layers and delivered to the site for transcription and replication. For herpesviruses, the DNA genome is delivered to the nucleus where DNA synthesis can occur.

HSV is an enveloped virus with glycoproteins on the surface of the envelope (2, 21). The glycoproteins are required for binding and entry into a host cell. There are attachment factors or receptors located on the outside of the cell (20). Some of these are just binding receptors that concentrate the virus on the cell's outer surface (21). Others are entry receptors that trigger the events that lead to membrane fusion. In HSV-1 infection, fusion occurs between the virion envelope and the plasma membrane (21). HSV-1 requires physiological pH to enter a cell (21). In order to get to the nucleus, viruses use nuclear localization signals (20). Because of the variety of cellular machinery available, it wants to be in the nucleus for replication. The capsid and some of the tegument protein move to the nuclear pores. There, the genome and some

proteins are delivered into the nucleus. Viral transcription, DNA replication, and packaging of the genome into capsids occur in the nucleus of the infected cell (5, 22). Transcription of the viral genes is regulated in a temporal and coordinated manner (3). This step in the infection process is termed macromolecular synthesis. Immediate early genes are the first set of genes to be expressed, and they activate early genes. Early genes include those genes that are necessary for viral DNA synthesis and, consequently, the replication of viral genome. Viral DNA synthesis occurs shortly after the appearance of the early gene products. Lastly, the late genes are transcribed. Their gene products make up the structural proteins of the virus. The packaged virus acquires its envelope upon egress from the nucleus (22). The first envelope is acquired from the inner membrane of the nucleus, but then it is lost. Later, the virus adds two layers called teguments. Then, these capsids bud into trans-Golgi vesicles which transport the prepared virus to membrane and out of the cell where it will spread and continue infection.

DNA replication is a necessary process that must occur in order for herpesviruses (and host cells) to replicate. Replication must be accurate to maintain genetic integrity (27). New strand synthesis occurs in a 5' to 3' fashion on the template strand of the DNA (30). The replication fork moves in one direction, but there are two strands being synthesized at a given time. One of these is the leading strand, and the other is the lagging strand. The leading strand is made continuously in the 5' to 3' direction, but, in order to synthesize DNA in the opposite direction of the fork's movement, the lagging strand must be discontinuous in short pieces called Okazaki fragments. This creates somewhat of a challenge because the DNA polymerase cannot initiate

synthesis, so it must be primed every time a new short piece is made. Also, the activities of both strands must be coordinated for efficient replication (24).

There are several key players required for DNA replication in most systems studied (27). These proteins make up the replisome. First, there must be a protein that recognizes the origin of replication and promotes some unwinding. Often these proteins are also responsible for the recruitment of the other proteins that make up the replisome machinery. A single stranded DNA binding protein is then needed to prevent the recently separated strands from reannealing and to provide some stability around the replication fork. A helicase is required to unwind the duplex DNA and promote fork progression. As already mentioned, a primer must be made since the polymerase can only extend a strand on a template. This role is usually filled by a primase, a specialized DNA dependent RNA polymerase. Of course, there must also be a DNA polymerase which is likely to have an editing function to increase fidelity. Polymerases often have a processivity factor which serves to keep the polymerase on the DNA. Finally, a nuclease activity of some sort is required to remove the primer, and a ligase is needed to seal the gap after the primer is removed and new DNA is laid down.

Genes Involved in HSV-1 Replication

To identify genes utilized in herpes simplex virus type 1 DNA replication, Wu et al. used a transient complementation DNA replication assay (6). Their goal was to identify genes that were necessary and sufficient for replication activity. The isolation of several virus mutants provided a basis for this experiment. These mutants exhibited little or no DNA synthesis. In this experiment, plasmids containing HSV genes were inserted into cells, and the cells were assayed for DNA synthesis. The study made use

of origin dependent synthesis, so each inserted plasmid also had an origin. They equipped every cell in the assay with a plasmid that included the genes that encoded two proteins that were already known to be required: polymerase and a DNA binding protein. There were three other plasmids that contained at least one unidentified trans gene each. Each plasmid was digested with a restriction enzyme, and the resulting fragments were assayed for their ability to compensate for the intact plasmid during replication. If a set of fragments was found that allowed DNA synthesis, each of the fragments was cloned. Then, the individual subclones were assayed alone and in combinations for their ability to supply the functions necessary for DNA replication. This method was repeated until the subclones contained a limited number of genes. At this point, the genes could be identified, cloned individually into a plasmid, and inserted into cells which could then be assayed for replication. The authors of this study identified five new genes that were necessary for successful DNA replication. Those five genes plus the previously found polymerase and DNA binding protein are necessary and sufficient for origin dependent DNA synthesis in HSV-1 (6).

The seven genes that are necessary and sufficient for HSV DNA replication are encoded by the virus's own genome (5). These genes encode an origin binding protein (UL9), a single stranded DNA binding protein (ICP8), a helicase-primase complex (UL5/UL8/UL52), a DNA polymerase (UL30), and a polymerase accessory protein (UL42) (3). The activity of these particular genes is best for optimal DNA synthesis. One of the proteins in some cases can be replaced by a homologue from another herpesvirus replication system though synthesis is usually less efficient (5). This suggests that there are key protein-protein interactions that aid in HSV DNA replication.

The UL30 gene encodes the DNA polymerase for the HSV system. The polymerase has 3'→5' exonuclease activity (3, 12). The exonuclease activity will remove incorrectly incorporated nucleotides, and thus it is the editing function (29). HSV-1 polymerase needs this function because it has low fidelity on its own compared to other polymerases. It will misincorporate a basepair every 1 in 300 pairs. Other polymerases like those of the bacteriophages T4 and T7 only misincorporate every 1 in 1000 and every 1 in 100,000 (29). The DNA polymerase encoded by UL30 has also been compared with polymerase delta of eukaryotic systems (12). It was found that the two have a similar amino acid sequence. They both also have two associated subunits that together make up the holoenzyme. Both are highly processive with their processivity factors and have 3'→5' exonuclease activities to increase fidelity. HSV-1 DNA polymerase interacts with the primer-template in a structural, rather than a sequence specific, manner (13). Polymerase alone is not processive, so it requires the help of its accessory protein, UL42 (5). UL42 is even required in abnormally high concentrations of polymerase for processive DNA synthesis (5). Ten times more of the catalytic subunit alone is needed to synthesize strands the same size as those made by the pol-UL42 complex (12). The presence of UL42 also improves the editing function and therefore increases fidelity (29).

UL42 is a double stranded DNA binding protein of around 60 kDa (11). It co-purifies with HSV polymerase which suggests a stable interaction exists between the two (11). This interaction greatly increases the processivity of polymerase on a single stranded DNA template. The UL42-polymerase complex forms without the activity of any other proteins (12). In infected cells, the two proteins form a 1:1 complex even

though UL42 is much more abundant than polymerase in these cells. The activity of UL42 depends on its ability to bind both to polymerase and to DNA (11). It has been proposed that UL42 acts as a sliding clamp similarly to the beta subunit of Pol III in *E. coli* and PCNA in eukaryotes (13). It increases the affinity of polymerase for the DNA substrate and also decreases the probability that polymerase will dissociate once it is bound to the DNA (13). It has been hypothesized that UL42, when bound to polymerase, will cause it to take on a more closed confirmation (28). This would account for the observed reduction in the dissociation rate and increased processivity. However, UL42 is not known to encircle the DNA like the other clamps (13). Because UL42 does not encircle the DNA, it does not need to be loaded either (28). The other clamps require a clamp loader and ATP to operate it. UL42 inherently binds to the DNA. The fact that UL42 is a double stranded rather than a single stranded DNA binding protein also makes it unique when compared to other processivity factors (28).

UL9 is a homodimer that recognizes a specific DNA sequence on the origin of replication (3, 7). It contains an ATP binding site that is conserved among origin binding proteins. It also has a 3'→5' helicase activity that is capable of unwinding long segments of DNA in the presence of a single stranded DNA binding protein like ICP8. Both the ATP binding site and the helicase activity are essential for viral DNA replication. HSV has three origins that are functionally equivalent, two within the short region repeats (oriS) and one within the long unique sequence (oriL) (6). UL9 binds cooperatively to one of these origin sequences by protein-protein interactions (7). It then forms a complex structure by winding the DNA around the proteins. UL9 also has

a hand in strand displacement synthesis (5). It interacts with polymerase through UL42 for this activity.

ICP8 is a single stranded DNA binding protein that adds some processivity to the helicase function of UL9 through a tight interaction (8). At the origin and throughout synthesis, ICP8 helps to preserve a single stranded DNA template by binding to the template DNA shortly after it has been unwound (8).

UL8, UL52, and UL5 together form the helicase-primase complex of Herpes Simplex Virus 1 (9). UL5 and UL52 exhibit all the enzymatic properties of the complex. The function of UL8 is not completely determined at this time, but it is essential *in vivo* for replication. UL8 increases the efficiency of the complex by stabilizing the interaction between the complex and the template. Also, UL8 may facilitate the entry of the complex into the nucleus of infected cells. UL52 contains motifs conserved throughout primases and UL5 contains those of helicases (3, 10). The two show a strong interdependence for DNA binding and enzymatic activities. The complex as a whole exhibits primase, 5'→3' helicase, and single stranded DNA dependent NTPase activities. For optimal activity, the helicase-primase complex needs a forked DNA substrate and ICP8. On a forked template, UL5 can be found nearest to the fork and UL52 on the single stranded DNA (3, 10).

In order to coordinate DNA replication, there are many events and interactions that must occur (3). Based on the *in vitro* activities of the HSV-1 replication proteins, it has been possible to produce a model for how the DNA replisome is assembled. When HSV-1 infects a cell, the DNA from the packaged virus enters the nucleus. Once the early gene products are made, DNA replication can begin. UL9 binds to the origin and,

together with ICP8, unwinds a portion of it. ICP8 binds to the newly single stranded DNA and prevents it from reannealing. The helicase-primase complex (UL52/UL5) is recruited, and it begins unwinding further. ICP8 binds the single stranded DNA and a primer is synthesized on the leading strand. Polymerase and UL42 are also recruited to the replisome (3). This recruitment is most likely directed by protein-protein and protein-DNA interactions. UL8 is thought to be responsible for keeping the replisome intact. It interacts with UL52/UL5, ICP8, and the polymerase which closely interacts with UL42. At the fork, two UL42-polymerase heterodimers would be predicted to be found along with one helicase-primase heterotrimer. Extension by polymerase-UL42 would be coupled to the unwinding of DNA by the helicase-primase since strand displacement by the polymerase complex is poor. The helicase-primase complex is translocated in a 5' to 3' manner along the lagging strand.

The Problem

Though numerous studies have been done and hypotheses have been made, it is still unclear exactly how all the processes are coordinated at the fork, particularly how leading strand and lagging strand synthesis are coordinated in vivo (23). It is not clear what proteins are needed to complete DNA replication of HSV-1 in vitro. No HSV-1 origin dependent DNA replication has been reconstituted in vitro using purified viral proteins (3). However, non-origin dependent replication has been accomplished (23). A minicircle template was used in this experiment, and replication progressed according to the rolling circle mechanism. The leading and lagging strands were labeled separately with labeled dNTPs so they could be distinguished. The leading strand contained a single labeled cytosine, and the lagging strand contained a single labeled

guanine to provide strand bias. In this study, ICP8, UL8, and UL9 were not required. However, it is known that these proteins are required in vivo (6). Also, the lagging strand synthesis completed here was not proven to be coordinated with the leading strand synthesis (23). It was less efficient, the fragments were longer than what is to be expected, and there was a significant lag time before any lagging strand activity was observed. This indicates that synthesis of DNA complementary to lagging strand synthesis was not observed. Furthermore, the fact that no origin dependent DNA replication has been accomplished in vitro indicates that there is something in the in vivo system that aids in replication. This is likely to be host proteins.

Hypothesis and Aims

Because complete replication has not been accomplished in vitro with the seven proteins that are known to be essential in vivo, it is hypothesized that other proteins, perhaps from the host cells, are also used by the virus. If that is the case, then the host proteins will be found interacting with the known proteins in infected cells. Such proteins can be identified by co-immunoprecipitation analysis using antibodies specific for a single essential HSV DNA replication protein or by analysis involving the interaction of the proteins with a tagged replication protein. Because of its specificity and availability in the laboratory, an antibody for the polymerase processivity factor, UL42, was used as well as a histidine tagged UL42 protein in the later experiments. The specific aims of this study were as follows:

- to alter the conditions for immunoprecipitation with UL42 antibody to a) allow quantitative removal of UL42 from extracts of HSV-infected cells; b) lower

stringency of wash conditions while retaining specificity in order to ensure that interactions of various strengths will be detected

- to use optimized conditions to scale up the experiment in order to identify proteins that are specifically immunoprecipitated by Mass Spectroscopy
- as an alternative approach based on unclear results, to attempt to identify interactions using a nickel column and a His-tagged UL42 protein.

Materials and Methods

Cells and Virus

Vero cells were grown overnight in culture medium Dulbecco's Modified Essential Medium (DMEM) containing 7.5% fetal bovine serum at 0.225% sodium bicarbonate at 34 °C in a 5% CO₂ atmosphere until confluent. The cells were infected with HSV-1 (strain KOS) at a multiplicity of infection (MOI) of 10 plaque forming units per cell. The cells were incubated with the virus in 5% CO₂ at 34 °C for 1 hour to allow for virus attachment. The inoculum was removed and 10 mL of culture medium was added to each plate. Cells were incubated in 5% CO₂ at 34 °C for 18 hours (one replication cycle) before they were collected by scraping and were pelleted by low speed centrifugation. The cells were washed with phosphate buffered saline (PBS) (2.7mM KCl, 1.5mM KH₂PO₄, 137mM NaCl, 8.1mM Na₂HPO₄, pH 7.4) without Ca and Mg twice. The cells were suspended in 1x Zweig's buffer (0.1M Tris-HCl pH 8.0, 10% glycerol, 0.5% Nonidet P-40, 0.5% Na deoxycholate, 150mM NaCl, and the protease inhibitors pepstatin A, leupeptin, aprotinin and phenylmethylsulphonyl fluoride at 1µg per mL) and incubated on ice for 10 minutes to allow for lysis. Extracts were clarified by centrifugation for 10 minutes at 4 °C and 15,000 rpm in a Sorvall RC-5B Refrigerated Superspeed Centrifuge. The supernatant contained the soluble protein and was retained for immunoprecipitation experiments by storage in aliquots at -80 °C. Mock infected extracts of Vero cells were prepared similarly except that no virus was added.

Co-immunoprecipitation

Cell extract (150 μ L) was combined with 350 μ L 1x Zweig's buffer. Sixty microliters of a rabbit monospecific antibody (834; Monahan et al., 1993) against UL42 was added to the mixture and gently agitated at 4°C for one hour. A Protein G Sepharose matrix (50 μ L of about 50% slurry) was washed four times with 200 μ L 1x Zweig's buffer. The agitated cell extract and antibody mix was added to the Protein G Sepharose (Amersham Pharmacia Biotech) matrix. This was agitated for one hour then centrifuged at 1000 rpm in an Eppendorf Centrifuge 5415C for 1 minute. The supernatant was collected as the unbound fraction. The matrix was washed with 200 μ L 1x Zweig's buffer four times. Following the last wash, the beads were boiled for 5 minutes in 25 μ L 1x Zweig's buffer and 30 μ L 2x loading buffer (0.125M Tris-HCl pH 6.8, 2% SDS, 20% Glycerol, 5% mercaptoethanol, 0.004% bromophenol blue) to elute and denature the bound proteins. The eluted material was subjected to electrophoresis at a constant 150V through a 10% polyacrylamide gel.

Visualization of Polypeptide Bands

A Coomassie stain was sometimes used to visualize the protein bands. Following electrophoresis, the gel was soaked in a glass dish in the gel-fixing solution (50% USP-grade 95% ethanol in water with 10% acetic acid) for 1 hour. This solution was removed by aspiration. The gel was then covered in the gel-washing solution (50% HPLC-grade methanol in water with 10% acetic acid) and incubated overnight at room temperature with gentle agitation. The dish was covered to avoid contaminations and evaporation of the solution. Following removal of the gel-washing solution, the gel was

soaked in the Coomassie stain (0.1% Coomassie blue R350, 20% methanol, and 10% acetic acid in water) at room temperature for 3 hours with gentle agitation. The stain was removed and the gel was soaked in the destain solution (50% HPLC-grade methanol in water with 10% acetic acid) for 30 minutes with gentle agitation. The destain solution was changed two more times after 30 minute intervals and the gel was allowed to agitate overnight in the destain solution. The destain solution was removed and the gel was placed in the storage solution (5% acetic acid in water) and scanned.

In come cases, bands were visualized by silver staining. Following electrophoresis, the gel was placed in a fixing solution (50% ethanol and 10% acetic acid in water) for 20 minutes with gentle agitation. The fixing solution was decanted and the gel was placed in the ethanol wash (30% ethanol in water) for 10 minutes. The ethanol wash was removed, and the gel was washed in ultrapure water for 10 minutes. The water was decanted, and the gel was soaked in the Sensitizer solution (Sigma, 1% ProteoSilver Sensitizer in water) for 10 minutes with gentle agitation. That solution was removed, and the gel was washed twice in ultrapure water for 10 minutes each time. The water was removed, and the Silver solution (Sigma, 1% ProteoSilver Silver Solution in water) was added to the gel and allowed to agitate for 10 minutes. The Silver solution was removed, and the gel was washed with ultrapure water for 1 minute. The water was decanted, and the gel was developed by adding the Developer solution (Sigma, 5% ProteoSilver Developer 1, 0.1% ProteoSilver Developer 2 in water) for 3 to 7 minutes. Upon development, the Stop solution (Sigma) was added and left for 5 minutes. The gel was scanned and stored in ultrapure water.

In some cases, bands were visualized by Lava Purple stain. Following electrophoresis, the gel was placed in fixation solution (15% ethanol in water and 10.1 g citric acid per liter) for 1 hour with gentle agitation. That solution was decanted, and the gel was added to the staining solution (6.3 g boric acid and 3.85 g sodium hydroxide per liter of water, 1:200 Lava Purple concentrate) for 2 hours with gentle agitation. The staining solution was removed, and the gel was soaked in the wash solution (15% ethanol in water) for 30 minutes with gentle agitation. The wash solution was removed, and the gel was placed in the fixing solution for 30 minutes. Gel was imaged by a fluorescence scan using a Typhoon scanner (GE Healthcare).

Western blotting

Following electrophoresis, the proteins in the gel were transferred to a nitrocellulose membrane at a constant 400mA for 1 hour. The membrane was blocked overnight in 3% gelatin in PBS with 0.05% NaN_3 at 4°C. The membrane was rinsed twice with Tris-buffered saline (TBS) (24.8mM Tris, 137mM NaCl, 5.1mM KCl, pH 7.4) and washed in TBS for 10 minutes with rotation. Antibody 834, directed to UL42, (1:500 dilution in PBS containing 1% gelatin) was added to membrane and incubated for 1.5 hours at room temperature with gentle agitation. The membrane was rinsed and washed for 10 minutes with TBS. Binding of antibodies to blots was detected by incubation with [^{125}I]-protein A (4 μCi in 1% gelatin in PBS) for 1.5 hours with agitation. The membrane was washed once again with TBS. The membrane was exposed to a storage phosphor screen overnight and analyzed using a Typhoon scanner (GE Healthcare).

Purification of His-UL42

His-UL42 was expressed in insect cells infected with recombinant baculovirus and purified by column chromatography. Sf9 insect cells were used to propagate recombinant baculoviruses. Cells were infected with His-UL42 recombinant at a multiplicity of infection of 5 PFU per cell. After 48 hours infection, cells were harvested and pelleted down at 4000 g for 15 min at 4 °C. The cell pellet was resuspended in Ni-NTA lysis buffer containing 50mM Na₂HPO₄, 300mM NaCl, 15% glycerol, and 10mM imidazole. The cell lysis mix was sonicated (10 sec with at least 5 sec intervals) until they were of liquid consistency. After sonication, the cell debris was spun down at 20,000 g for 30 min at 4 °C. The supernatant was collected and the pellet was discarded. Ni-NTA agarose column (5 mL) was equilibrated in Ni-NTA lysis buffer. Cell lysate was loaded into the sample loop and injected onto the column manually. The column was washed with Ni-NTA wash buffer which contains 50mM Na₂HPO₄, 300mM NaCl, 15% glycerol, and 20mM imidazole at 10 column volumes. The proteins on the column were eluted off by increasing the imidazole concentration to 300mM. His-UL42 was concentrated by Centricon YM50.

Precipitation with His- UL42

Cell extract (100µL) was combined with 30 µL of purified histidine tagged UL42 and 370µL of Ni-NTA lysis buffer (50mM NaH₂PO₄, 150mM NaCl, 15% glycerol, 10mM imidazole) and agitated overnight at 4 °C. The mixture was then added to 100 µL Ni-NTA agarose beads (Qiagen) which were washed previously five times in 200 µL Ni-

NTA lysis buffer. The mixture was agitated 4 hours to allow for binding then centrifuged at 1000 rpm in an Eppendorf Centrifuge 5415C for 1 minute. The supernatant was collected as the unbound fraction. The matrix was washed with 200 μ L Ni-NTA lysis buffer four times. The wash fractions were also collected. Fifty microliters of Ni-NTA elution buffer (50mM NaH_2PO_4 , 150mM NaCl, 15% glycerol, 300mM imidazole) was added to the matrix and incubated for 5 minutes. The eluted material (50%) was subjected to electrophoresis at a constant 150V through a polyacrylamide gel. The results were analyzed by silver stain or Coomassie stain.

Results

Optimizing Conditions for Immunoprecipitation

Different batches of infected cell extracts were analyzed by Western blot to select the extracts with the greatest amount of UL42 for future immunoprecipitations. The extracts with the strongest signal (lane 4) were used in the remaining experiments. The Western blot demonstrated the specificity of the antibody and showed no cross-reactivity with mock-infected extracts (Figure 1).

Conditions were varied to optimize the amount of UL42 that could be immunoprecipitated and the number and amount of the proteins with which UL42 interacts. Previous work was performed in the lab indicating that the amount of antibody was not limiting under the reaction conditions. The first condition varied was the amount of Protein G sepharose added to the cell extract and antibody to ensure that the matrix amount was not limiting. As observed by the Western blot, the amount of UL42 did not change as the quantity of Protein G sepharose matrix was increased (Figure 2). Therefore, I selected 50 μ L of Protein G sepharose slurry for the remaining experiments.

The remaining conditions were varied by altering the Zweig's buffer used throughout the experiment. I tested the effect of increasing salt concentrations on the specificity and efficiency of immunoprecipitation. Interactions of host proteins with UL42 that are weak might be abolished in high salt. On the other hand, low salt conditions could leave nonspecific interactions. Many protein-protein interactions are ionic, and therefore, the salt ions compete with the proteins for binding. The salt stringency was varied by changes in the concentration of NaCl added to the Zweig's buffer. NaCl at 50,

75, 100, and 150mM concentrations were all tested. There was no significant difference in the amount of UL42 pulled down with any of the salt concentrations (Figure 3A). Comparison of the input and the immunoprecipitated lanes in the Western blot, demonstrates that UL42 was not being quantitatively immunoprecipitated despite low salt concentrations (Figure 3A). The input lane (lane 1) represents only one-fifth that used in the immunoprecipitations, yet it still shows significantly more UL42 on a Western blot. No bands corresponding to UL42 were visible in the silver stain (Figure 3B).

Detergents also have an effect on protein-protein interactions. Detergents provide competition for hydrophobic interactions that sometimes occur between proteins. There are two detergents in the Zweig's buffer (0.5% Na-deoxycholate and 0.5% NP-40). I tested the efficiency of immunoprecipitations when one of the detergents was omitted from the Zweig's buffer. The Western blot shows that more UL42 is pulled down when NP-40 was absent from the immunoprecipitation buffer and the washes (Figure 4A). Some light bands are also visible on the silver stain (Figure 4B). Therefore, Zweig's buffer without NP-40 was used for the remainder of the experiments. However, it was quite apparent at this point that immunoprecipitations were inefficient at pulling down UL42 under all the tested conditions.

For the next immunoprecipitation, 0.5% SDS was added to the Zweig's buffer. Small amounts of SDS would cause mild or partial unfolding of the proteins. If the epitope recognized by the antibody is partially buried, SDS would be expected to increase the amount of UL42 immunoprecipitated. The Western blot detected higher levels of UL42 when SDS was used (Figure 5A). The silver stain also had bands

throughout the lane (lane 6) when SDS was used (Figure 5B). Because SDS did increase the efficiency of UL42 immunoprecipitation with the 834 antibody, it was included in the remaining experiments. Bands on the Western blot were more defined in the sample containing SDS. Also, the silver stain showed more protein in general when SDS was used.

To better separate polypeptides and to obtain sufficient material for submission for mass spectrophotometric analysis, immunoprecipitations of infected and mock extracts were scaled up, and the immunoprecipitates were separated by electrophoresis through a 5-15% polyacrylamide gel. The gel was analyzed using a Coomassie blue stain because that can be submitted for mass spec analysis. Upon examination of the gel, no bands appeared to be unique to immunoprecipitates of the infected cell extracts compared to those from mock-infected extracts. The mock infected extracts were used to detect nonspecific interactions with the protein G sepharose matrix.

It had been suggested that a more sensitive stain would provide clearer results that could be analyzed by mass spec. After consultation with the mass spec department, a lava purple stain was used along with a silver stain for comparison. Lava purple is comparable in sensitivity to a silver stain, and polypeptides stained by the Lava purple protocol are amenable to mass spec analysis (Figures 6A and 6B). However, there were still no observable differences between the immunoprecipitated mock and infected cell extracts.

Two different collections of mock infected cells were used to confirm that there was nothing wrong with one particular batch of cells. A Western blot was run to ensure that UL42 was not detected in any of the mock infected samples. UL42 was only

observed in the infected cell extract (data not shown). Also, one mock infected extract was immunoprecipitated without SDS in the Zweig's buffer to see if that would account for the bands observed in the mock lanes previously. As seen in the Coomassie stain, the addition of SDS made no difference, and all the bands observed in the mock immunoprecipitations analyzed appeared identical to those in the infected cell extract immunoprecipitation (Figure 7).

Despite these optimizations, UL42 was still not being quantitatively immunoprecipitated. A final immunoprecipitation was completed and analyzed by a lava purple stain. The immunoprecipitates from infected cells were once again not distinguishable from the immunoprecipitates from the mock infected sample (Figure 8). I concluded that immunoprecipitation, at least with this antibody, was not an effective way to isolate partners that interact with UL42.

Histidine-tagged UL42

It was thought that the use of a purified histidine tagged UL42 protein might give more efficient precipitations. Using a purified protein gives one control over the abundance of the protein. A histidine tagged protein allows for specific binding to a Nickel column. The elution is controlled with the use of a high concentration of imidazole (300mM). As shown in Figure 9, the his-tagged UL42 used in this experiment was purified and concentrated to give a defined band.

Cell extracts were mixed with His-UL42 then allowed to bind to a nickel column. Ni-NTA lysis buffer was used during the binding of the tagged protein (His-UL42) to the column and for all the washes. The salt concentrations in this buffer were varied between 50 and 150mM. The elution for the infected cell extract showed many protein

bands, some of which were unique from the mock infected extract elution (Figure 10). It was thought that the results using a 150mM NaCl concentration had more protein in some bands, so 150mM was used in the remaining experiment.

Because the bands were well defined and numerous, it was decided that scaling up the experiment and using a gradient gel may provide results that could be submitted to mass spec for identification. Infected and mock extracts were incubated with and without his-UL42. The extracts incubated without his-UL42 serve as a control for nonspecific binding of protein to the Ni-NTA agarose beads. The proteins eluted from the beads were subjected to electrophoresis on a 5-15% gradient gel. The gel was analyzed using a Coomassie blue stain because that can be submitted to mass spec. Elutes obtained from the mock infected and infected extracts incubated without his-UL42 were compared to elutes obtained from mock infected and infected extracts incubated with his-UL42. The protein bands in the lanes containing elute incubated without his-UL42 represent nonspecific interactions with the Ni-NTA matrix (Figure 11, lanes 10 and 11). None of the protein bands observed on the gel were unique to the infected extract elution (Figure 11, lane 13). This procedure is also not clearly detecting the proteins that are interacting with UL42. In order to reduce the background observed in this experiment, it may be necessary to do more washes or increase the salt stringency of the washes.

Discussion

The aim of this study was to identify proteins that interact with UL42, the polymerase accessory protein, and the rest of the viral replisome. It was thought that these unidentified proteins could be the missing link to reconstituting the viral DNA replication system in vitro. There were two techniques used to attempt to identify the proteins. The first was co-immunoprecipitation using an antibody against UL42. The experiment turned out to be inefficient at precipitating UL42 and other interacting proteins. In some cases, the identity of protein bands was confirmed by Western blotting using an antibody to detect the presence of UL42. As seen in the each Western blot, UL42 was not quantitatively pulled down using this antibody. It was possible in the earlier experiments that some protein was lost due to human error. Some of the matrix may have been removed accidentally with the washes. However, by the later experiments, more care and developed skill were able to prevent much loss.

Throughout the immunoprecipitations, it was determined that the antibody may not be acting as efficiently as one would like. SDS is a detergent that relaxes a protein's conformation and helps denature it. When SDS was added to the buffer used during the initial binding step, the efficiency of the immunoprecipitation improved. More UL42 was detected on the Western blot, and more protein was observed on the silver stain. This suggested that the epitope to the antibody may be somewhat buried when UL42 is in its natural conformation. The SDS relaxed the conformation of UL42, it most likely exposed the epitope, and allowed more antibody to bind, which in turn led to improved efficiency for immunoprecipitating UL42.

The other technique used in this study involved the use of histidine tagged UL42. The purified tagged protein was added to cell lysate and allowed to bind to the proteins within the lysate. For UL42 to interact with proteins present in the mock-infected extracts, the interactions must not be dependent upon the presence of other viral proteins or a functional viral DNA replisome. In infected cell extracts, these other proteins would be present. However, it is possible that they might not be able to interact with an exogenous protein when they are already interacting with partners in the infected cell. In the future, it may be beneficial to express his-tagged UL42 *in vivo* so that interactions can occur in the context of active viral DNA replication. To do this, the his-tagged UL42 could be expressed in vero cells that are infected with a HSV-1 strain that is mutant for UL42. The his-tagged UL42 would complement the null mutant (ref. Thornton et al., 2000) and be allowed to interact with proteins in a natural environment.

Non specific interactions with the matrix could also have accounted for some of the inefficiency. In immunoprecipitations, Protein G will bind to antigen-antibody complexes, but there could be nonspecific interactions of the protein G or the sepharose support with the extracts. To account for these, mock infected extracts were prepared and immunoprecipitated with the infected extracts. During the his-tagged UL42 experiments, both mock infected and infected cell lysate were incubated in the absence of his-tag UL42 and then were allowed to bind to the Ni-NTA agarose beads. This way, any proteins that bound nonspecifically to the matrix could be observed. It was demonstrated that under our conditions, a large number of proteins could bind to the Ni-NTA agarose matrix even in the absence of his-UL42 (Fig. 11). The presence of so many non-specific bands prevents an accurate assessment of polypeptide species

capable of uniquely interacting with UL42. Increasing the number of washes could help to decrease the number of nonspecific interactions with the matrix. It may also help to increase the salt stringency of the wash since I used only 150 mM NaCl. If the salt concentrations were higher, then the amount of nonspecific protein interactions would be expected to decrease.

It is also possible that UL42 is not the best candidate for determining proteins that interact with the DNA replication complex. Another viral DNA replication protein may need to be used. Alternatively, the antibody selected for immunoprecipitation with UL42 was not optimum, particularly since evidence suggests that the epitope is at least partially inaccessible (Fig. 5A). Nevertheless, HSV-1 DNA replication proteins should continue to be studied because they could be important in the development of antiviral medications. HSV-1 is a great model system whose resources can be utilized in battles against herpesviruses.

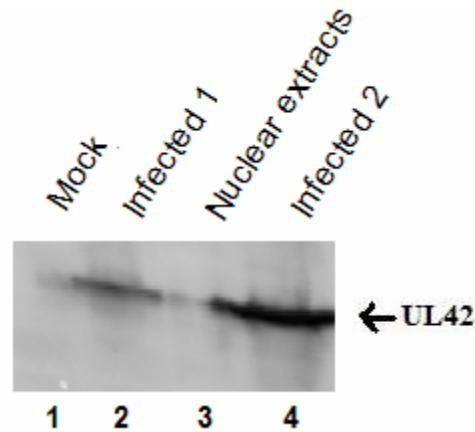


Figure 1

Western blot detecting UL42. Vero cells were infected with HSV-1 at a MOI of 10 or were mock-infected and total cellular (lanes 1, 2, 4) or nuclear extracts (lane 3) were prepared from cells harvested at 18 hr post-infection. UL42 was detected using specific anti-peptide antibody to UL42 as the primary antibody followed by [125 I] –Protein A. The blot was exposed to a storage phosphor screen for 24 hr and scanned with a Typhoon image analyzer.

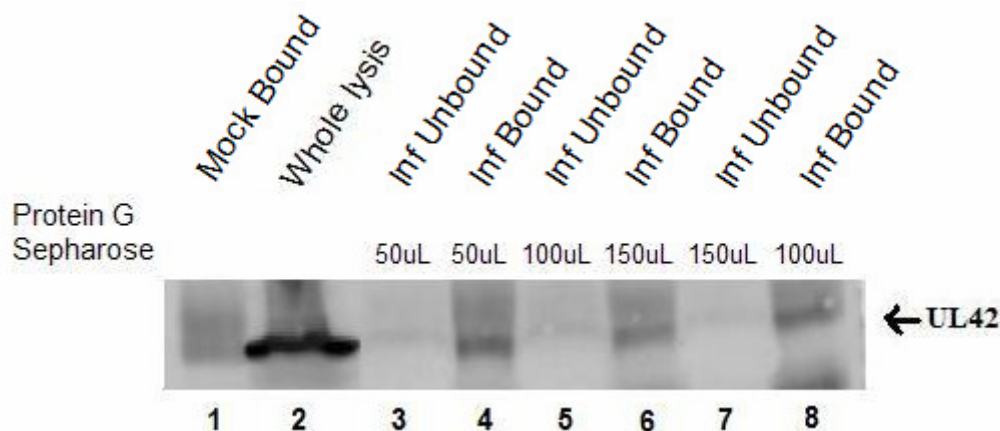


Figure 2

Effect of Protein G Sepharose amount on efficiency of immunoprecipitation of UL42 with antibody 834. A constant amount (150ul) of infected cell lysate was incubated with 60ul of anti-UL42 antibody for 1 hr as described in Materials and Methods. Increasing amounts of a 50% slurry of Protein G Sepharose (50-150 ul) was added to precipitate antigen-antibody complexes. A portion of the unbound (4%) and bound (50%) was separated by electrophoresis through 10% polyacrylamide gels and Western blotted with UL42-specific antibody as described in the legend to Fig. 1. Lane 2 represents 20% of the amount immunoprecipitated from infected cell extracts. Lane 1 represents mock infected extracts subjected to the procedure above using 50uL Protein G Sepharose slurry.

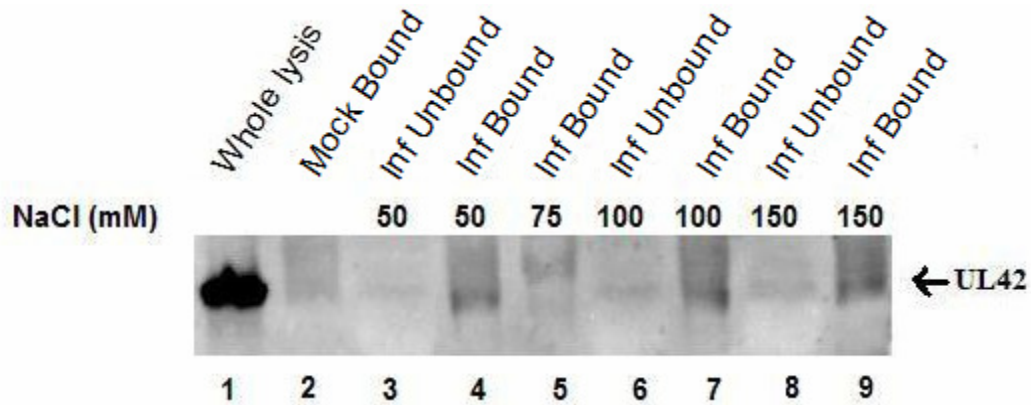


Figure 3A

Effect of NaCl concentration on efficiency of immunoprecipitation of UL42 with antibody 834.

A constant amount (150ul) of infected cell lysate was incubated with 60ul of anti-UL42 antibody for 1 hr, and 50ul of 50% slurry of Protein G Sepharose was added to precipitate antigen-antibody complexes as described in Materials and Methods. The NaCl concentration in the buffer used to wash the matrix was from 50 to 150mM. Bound and unbound fractions were analyzed by electrophoresis as described in the legend to Fig. 2 and UL42 was detected by Western blot with UL42-specific antibody as described in Fig. 1. Lane 2 represents 20% of the amount immunoprecipitated from infected cell extracts. Lane 1 represents mock infected extracts subjected to the procedure above using 150mM NaCl.

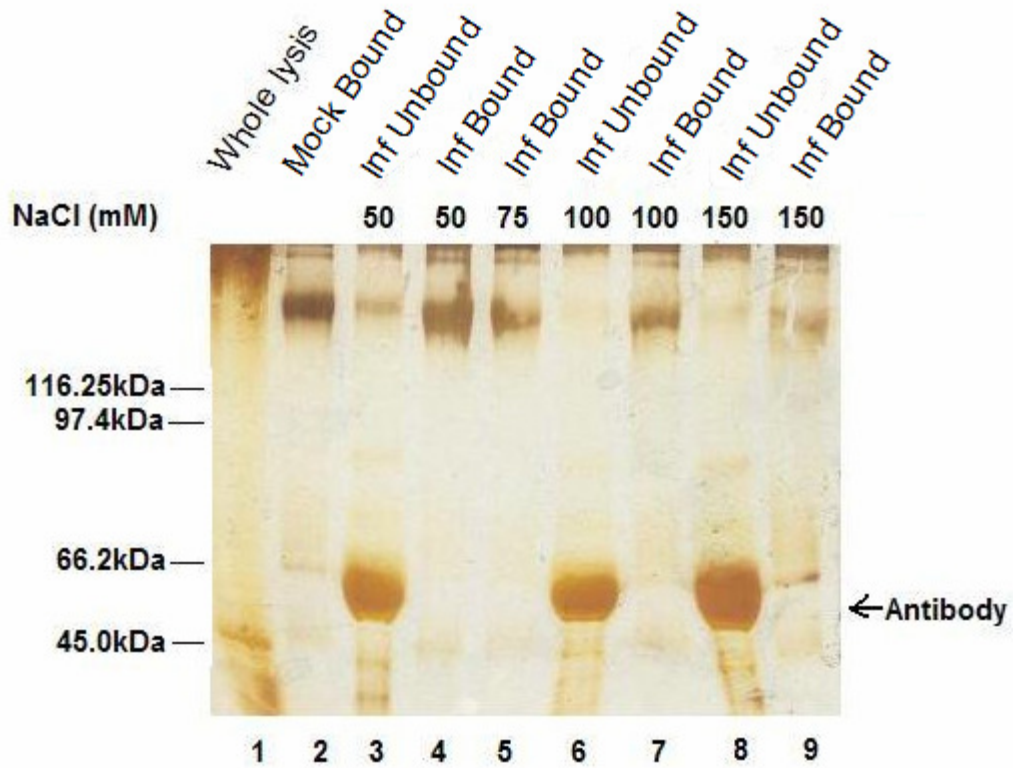


Figure 3B
Effect of NaCl concentration on efficiency of immunoprecipitation of UL42 with antibody 834. Fractions as described in the legend to Fig. 3A were separated by electrophoresis and the gel was silver stained.

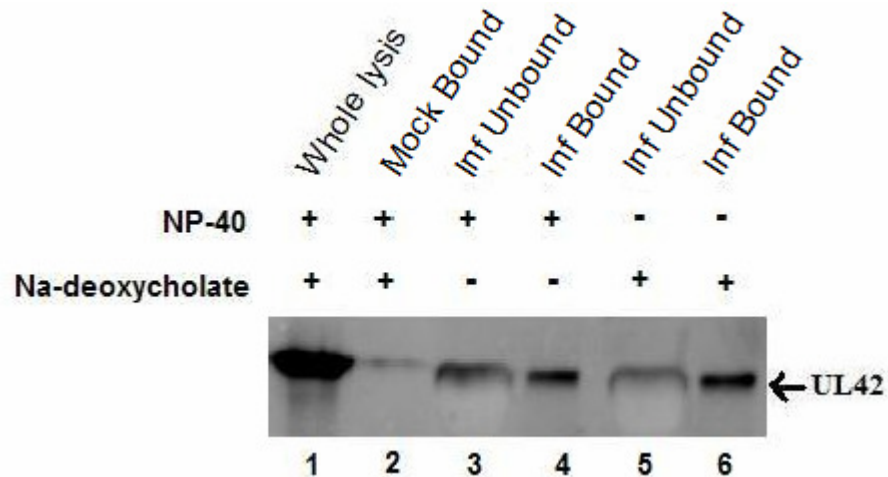


Figure 4A

Effect of varying detergents in Zweig's buffer on efficiency of immunoprecipitation of UL42 with antibody 834.

A constant amount (150ul) of infected cell lysate was incubated with 60ul of anti-UL42 antibody for 1 hr, and 50ul of 50% slurry of Protein G Sepharose was added to precipitate antigen antibody complexes as described in Materials and Methods. The detergents (NP-40 at 0.5% and Na-deoxycholate at 0.5%) in the Zweig's buffer used to wash the matrix were included as noted on the figure. Input, bound and unbound fractions were separated by electrophoresis as described in the legend to Fig. 2 and a Western blot with UL42-specific antibody as described in Fig. 1. Lane 1 represents 20% of the amount immunoprecipitated from infected cell extracts. Lane 2 represents mock infected extracts subjected to the procedure above using both NP-40 and Na-deoxycholate.

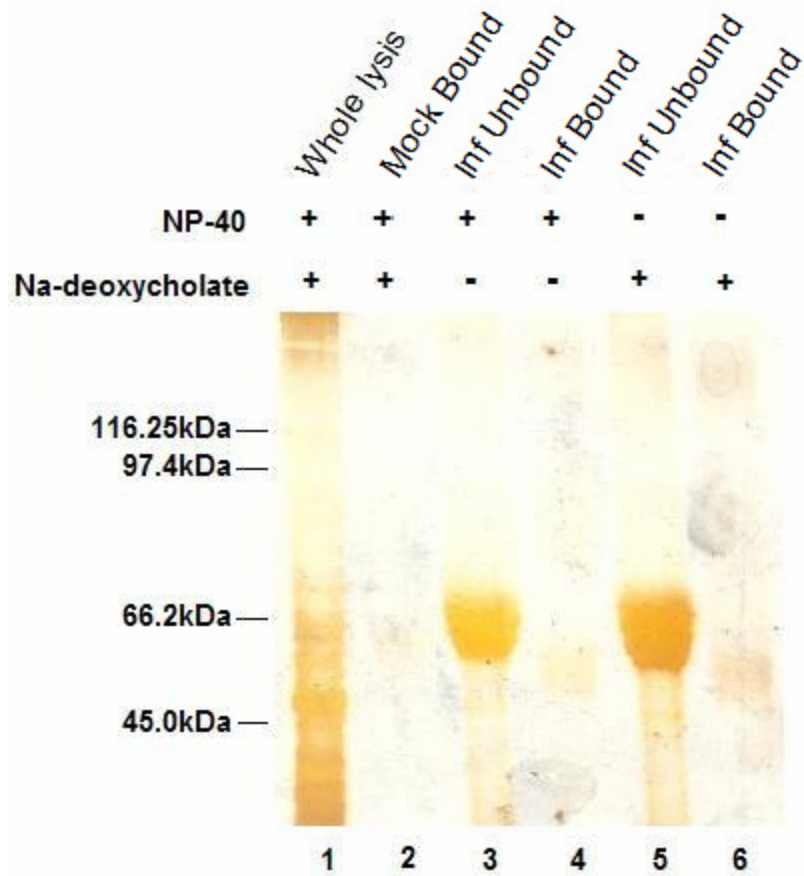


Figure 4B

Effect of varying detergents in Zweig's buffer on efficiency of immunoprecipitation of UL42 with antibody 834. Fractions from the experiment described in Fig. 4A were separated by polyacrylamide gel electrophoresis and the gel was fixed and silver-stained.

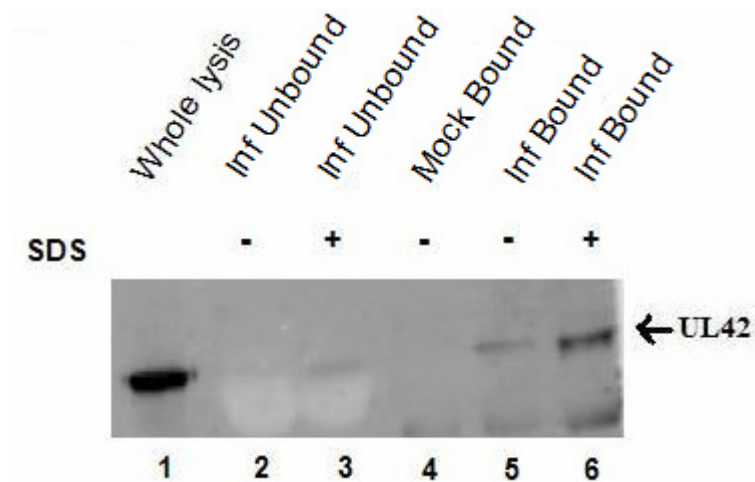


Figure 5A

Effect of SDS on efficiency of immunoprecipitation of UL42 with antibody 834. A constant amount (150ul) of infected cell lysate was incubated with 60ul of anti-UL42 antibody for 1 hr, and 50ul of 50% slurry of Protein G Sepharose was added to precipitate antigen antibody complexes as described in Materials and Methods. SDS was added to the buffer to a final concentration of 0.5% and was used to wash the matrix. Input, bound and unbound fractions were analyzed by electrophoresis as described in the legend to Fig. 2 and a Western blot with UL42-specific antibody as described in Fig. 1. Lane 1 represents 20% of the amount immunoprecipitated from infected cell extracts. Lane 4 represents mock infected extracts subjected to the procedure above.

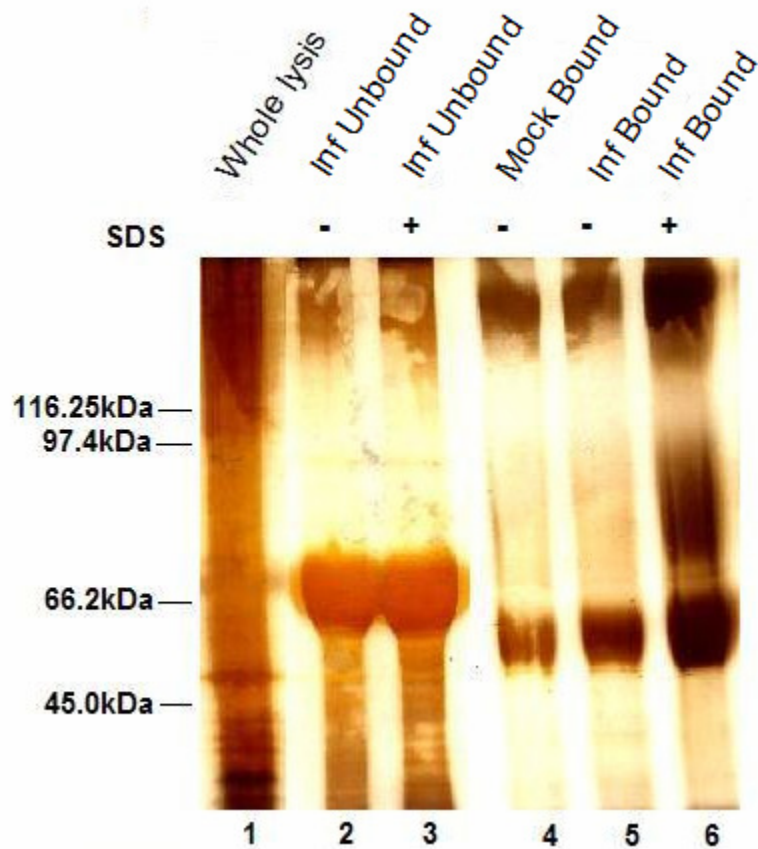


Figure 5B

Effect of adding SDS on efficiency of immunoprecipitation of UL42 with antibody 834. Fractions from the experiment described in Fig. 4A were separated on a polyacrylamide gel and analyzed by silver staining.

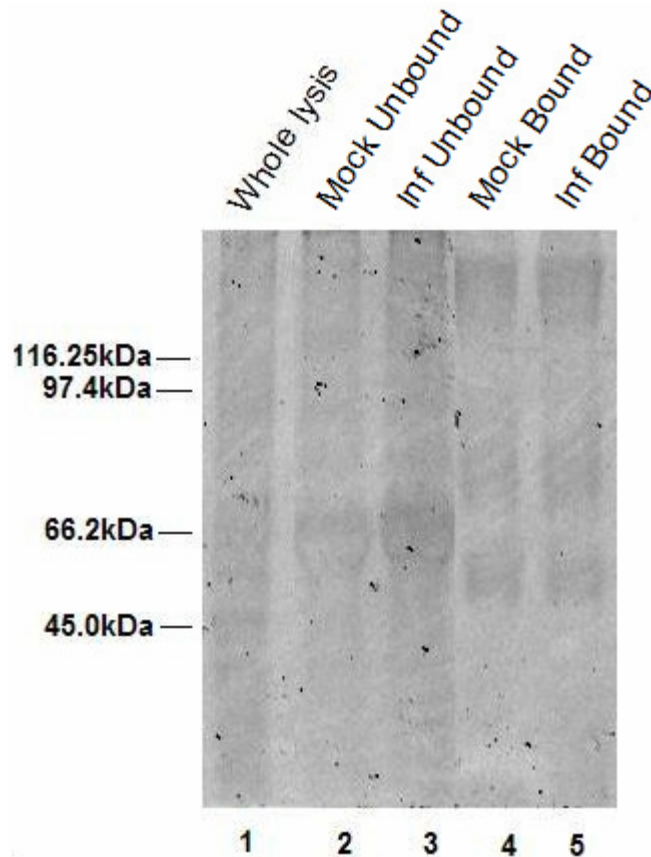


Figure 6A

Comparing sensitivity of Lava Purple stain to a silver stain. A constant amount (150ul) of infected cell lysate was incubated with 60ul of anti-UL42 antibody for 1 hr, and 50ul of 50% slurry of Protein G Sepharose was added to precipitate antigen antibody complexes as described in Materials and Methods. The results were analyzed by electrophoresis as described in the legend to Fig. 2, and a Lava Purple stain was used to visualize the bands under UV light with the aid of the Typhoon image analyzer.

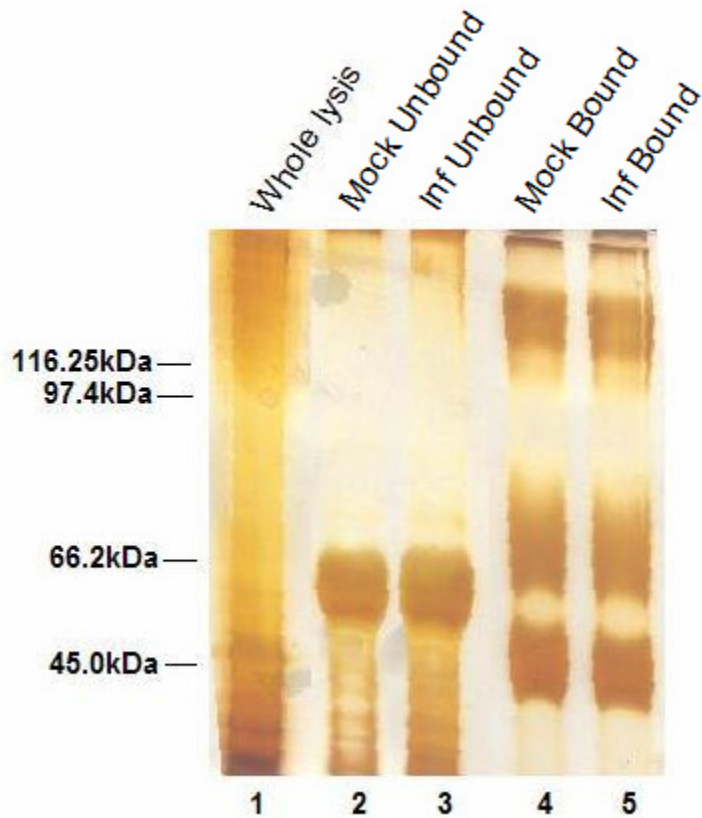


Figure 6B

Comparing sensitivity of Lava Purple stain to a silver stain. Experiment is the same as that noted in Fig. 6A, but the gel was silver stained.

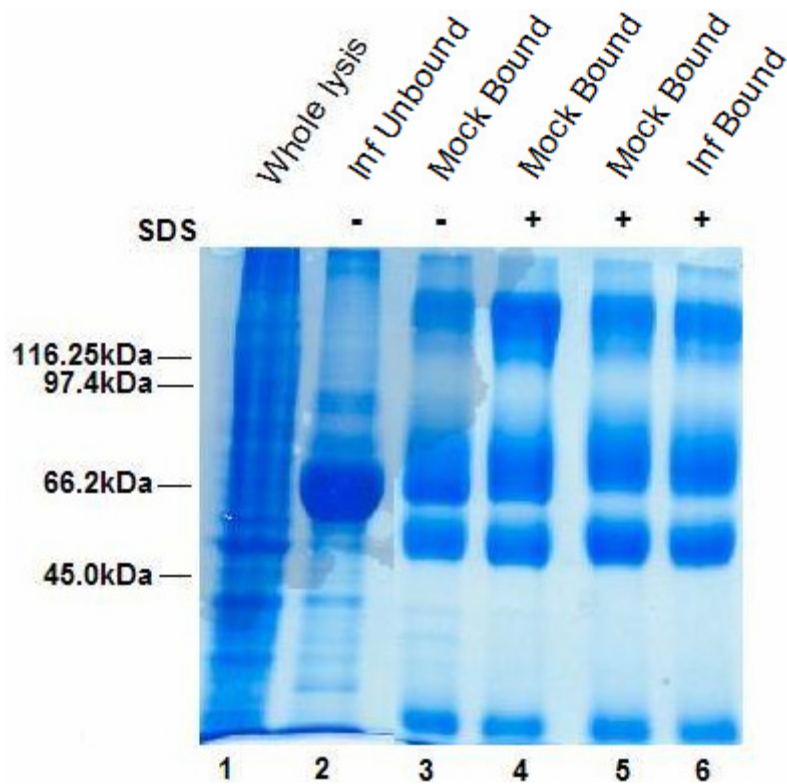


Figure 7

Effect of 0.5% SDS in Zweig's buffer on mock extracts in an immunoprecipitation of UL42 with antibody 834. Experiment is the same as that noted in Fig. 6A except that gels were stained with Coomassie blue. 0.5% SDS was added to the Zweig's buffer used throughout the immunoprecipitation as noted.

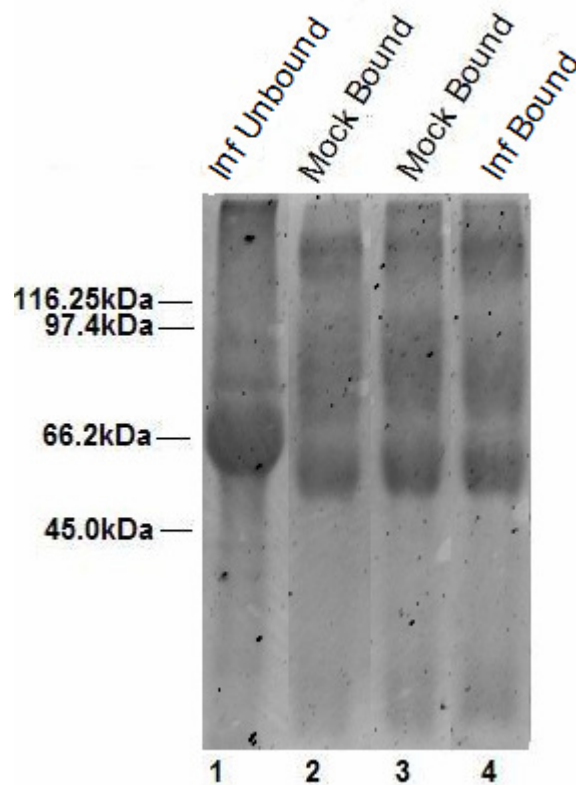


Figure 8

Lave purple stain after immunoprecipitation of UL42 by antibody 834. A constant amount (150ul) of infected cell lysate was incubated with 60ul of anti-UL42 antibody for 1 hr, and 50ul of 50% slurry of Protein G Sepharose was added to precipitate antigen antibody complexes as described in Materials and Methods. The results were analyzed by electrophoresis as described in the legend to Fig. 2, and a Lava Purple stain was used to visualize the bands under UV light.

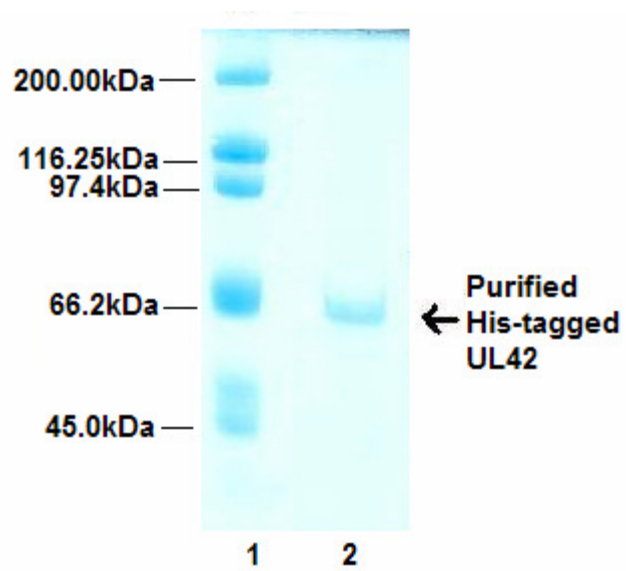


Figure 9
Purification of histidine tagged UL42. The Histidine tagged UL42 was checked for purification (Lane 2). In this case, 20ul of the purified protein was loaded.

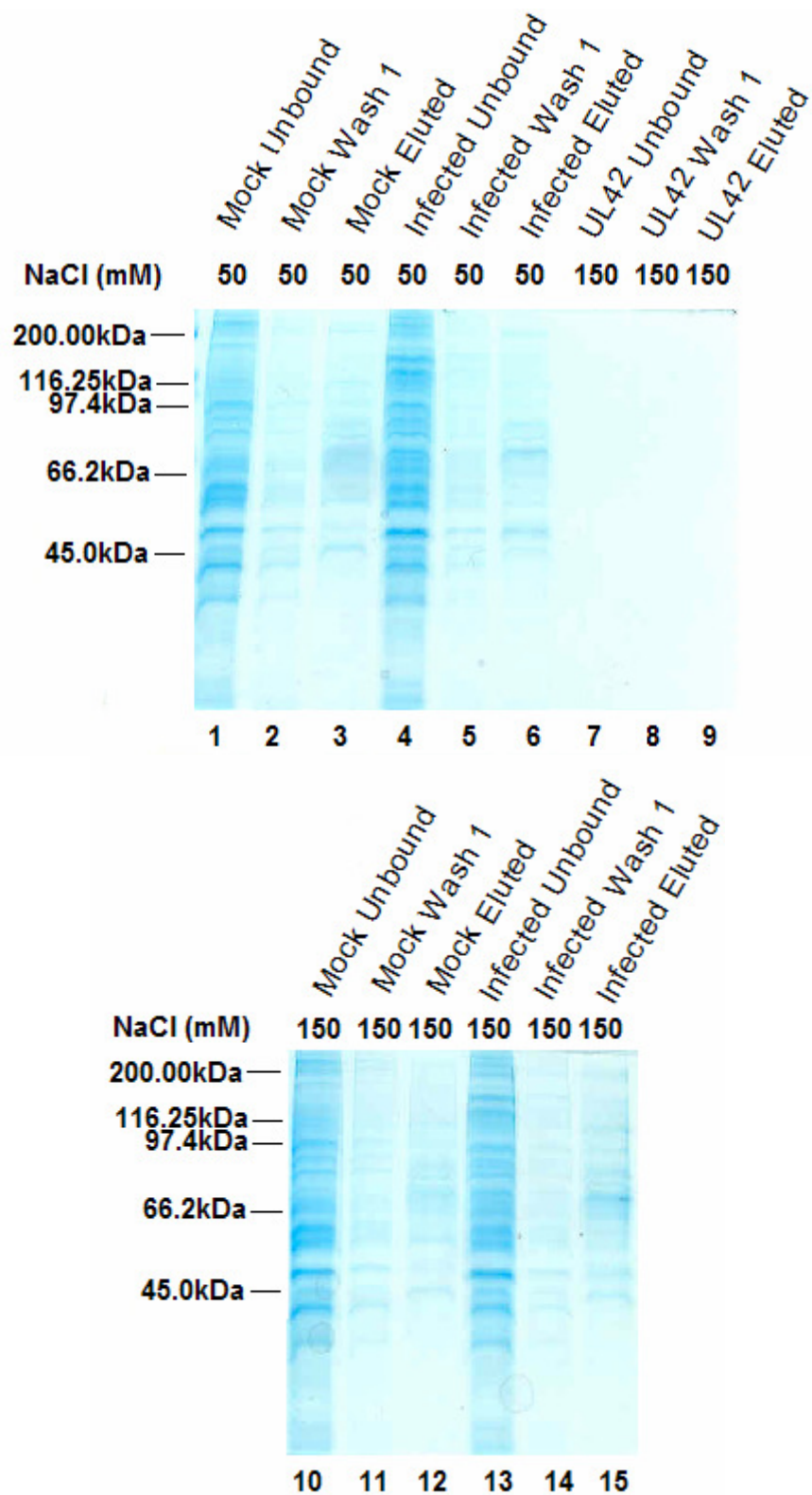


Figure 10
Using Histidine tagged UL42 to pull down associated proteins. Purified His-tagged UL42 was incubated with 100ul of either infected or mock cell lysate overnight. Ni-NTA

agarose beads were used to precipitate his-tagged UL42 and any proteins interacting with it as described in Materials and Methods. The NaCl concentration in the Ni-NTA buffer used in the washes was varied. The results were analyzed by polyacrylamide (10%) gel electrophoresis as described in the legend to Fig. 2 the gel was stained with Coomassie blue to visualize the proteins. Lanes 7, 8, and 9 contain his-tagged UL42 incubated without any cell lysate.

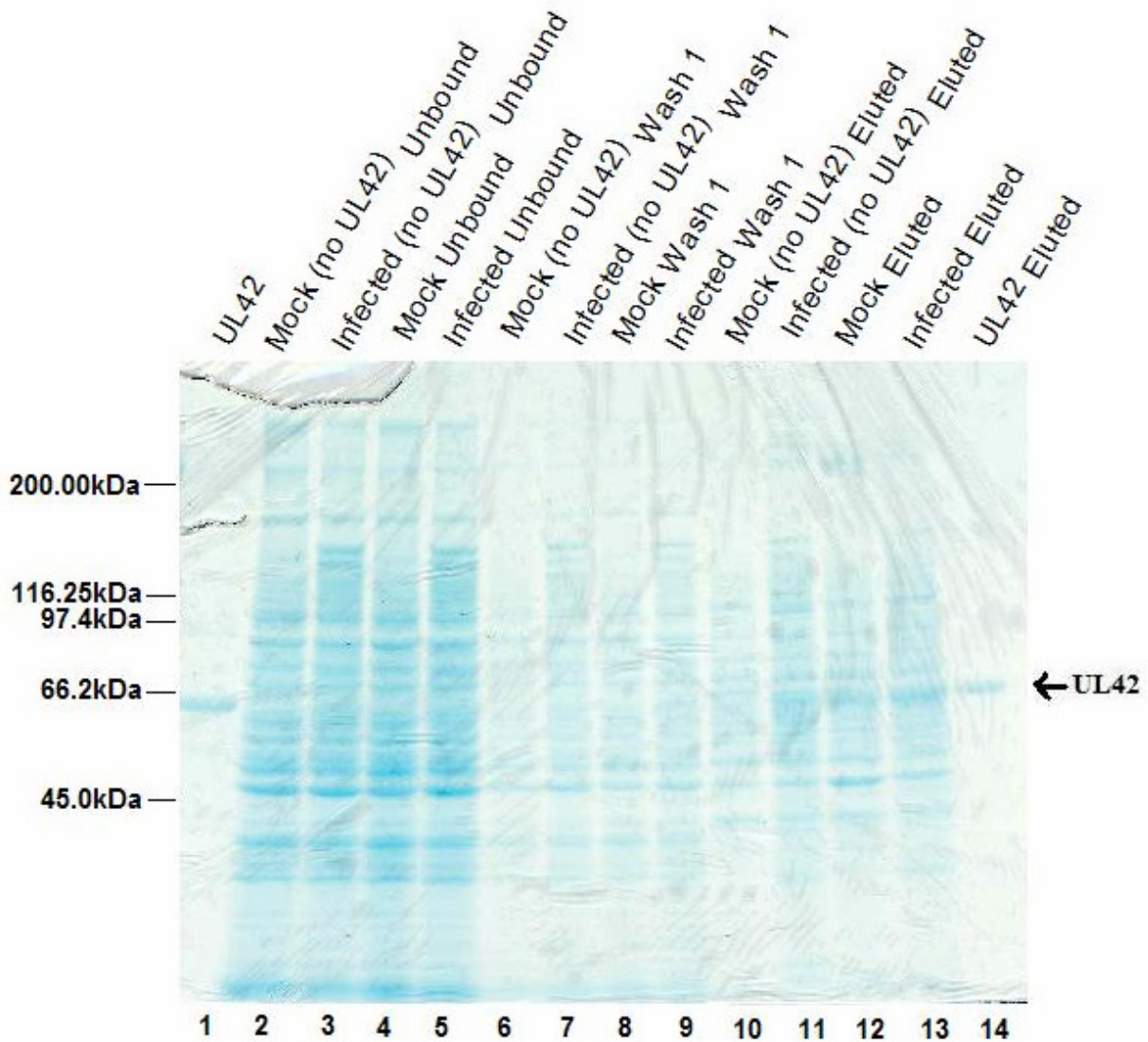


Figure 11
A scaled up experiment using Histidine tagged UL42 to pull down associated proteins. Ni-NTA agarose was used to pull down his-UL42 and associated proteins as described above except that for this experiment a 150mM concentration of NaCl was used in the wash buffer.

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